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Effect of Pneumococcal Vaccination on Nasopharyngeal Carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* in Fijian Children

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The 7-valent pneumococcal conjugate vaccine (PCV7) reduces carriage of vaccine type *Streptococcus pneumoniae* but leads to replacement by nonvaccine serotypes and may affect carriage of other respiratory pathogens. We investigated nasopharyngeal carriage of *S. pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* in Fijian infants participating in a pneumococcal vaccine trial using quantitative PCR. Vaccination did not affect pathogen carriage rates or densities, whereas significant differences between the two major ethnic groups were observed.

Nasopharyngeal (NP) carriage of pathogenic bacteria is the primary reservoir for maintaining bacterial species within a population (7) and is considered a prerequisite for development of major childhood diseases, including bacterial pneumonia, meningitis, and otitis media. *Streptococcus pneumoniae* is the most common bacterial cause of childhood pneumonia and is responsible for at least 800,000 child deaths annually, primarily in developing countries (18, 33). While rarely fatal, otitis media is the most frequently reported childhood bacterial infection: approximately 80% of children experience otitis media by age three (17). *S. pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are the predominant causes of otitis media (36).

PCV7 (Prevnar; Pfizer Inc.) effectively reduces carriage and subsequent disease caused by the serotypes of *S. pneumoniae* included in the vaccine. However, pneumococcal vaccination has minimal impact on the overall rate of pneumococcal carriage due to replacement by nonvaccine serotypes (3, 8, 11). Reports demonstrating an inverse relationship between nasopharyngeal carriage of vaccine type *S. pneumoniae* and *Staphylococcus aureus* (2, 21, 22) and increases in the proportion of otitis media caused by nontypeable *H. influenzae* following PCV7 vaccination (1, 4, 41) generated concern that removal of vaccine type pneumococci from the nasopharynx could facilitate colonization by other respiratory pathogens. This study aimed to evaluate the effects of pneumococcal immunization on carriage of respiratory pathogens in a high-risk population.

A quantitative real-time PCR (qPCR) method was developed to measure *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *S. aureus* and applied to nasopharyngeal swabs collected from 17-month-old participants in a phase II pneumococcal vaccine trial in Suva, Fiji. The trial investigated appropriate infant dosing strategy of PCV7 and effects of a 23-valent polysaccharide (23vPPS; Pneumovax; Merck & Co., Inc.) booster given at 12 months. Enrollment, ethical approval, and swab collection and storage have been detailed previously (25, 27, 29). Swabs (collected in 2006 and 2007) in STGG media (19) were transported to Melbourne, Australia, on dry ice and stored at −80°C until use. The following sample groups were examined: group A ($n = 54$), who received three doses of PCV7 at 6, 10, and 14 weeks of age; group B ($n = 48$), who received three PCV7 doses plus a booster of 23vPPS at 12 months of age; and group H ($n = 59$), who were unvaccinated. Previous examination of pneumococcal serotypes showed that three doses of PCV7 led to a reduction in carriage of vaccine type *S. pneumoniae* that was sustained to the age of 17 months, whereas carriage rates of non-PCV serotypes were similar, and that 23vPPS had no impact on carriage (27).

Cells from a 100-μl aliquot of sample were lysed in 200 μl of 50 mM phosphate buffer, pH 6.7, containing 1 mg/ml lysozyme, 0.075 mg/ml mutanolysin, and 2 mg/ml proteinase K and incubated at 56°C for 45 min. An additional 2 mg/ml proteinase K and 1% SDS were added, and then cells were incubated at 56°C for 10 min. DNA was extracted and purified into 100 μl elution buffer using the QIAmp DNA minikit (Qiagen). DNA for standard curves was extracted from *S. pneumoniae* ATCC 6305, *H. influenzae* F412 (kindly provided by Cynthia Whitchurch, University of Technology, Sydney, Australia), *S. aureus* ATCC 29213, and *M. catarrhalis* ATCC 8176. We developed two quantitative duplex PCR assays, one to detect *S. pneumoniae* and *S. aureus* and another to detect *H. influenzae* and *M. catarrhalis*. *S. pneumoniae* was detected using previously published primers and probes (30). Primers for *S. aureus* detection were modified for use with a previously published probe (6) for enhanced sensitivity. Sequence alignment of *M. catarrhalis copB* from 14 available GenBank entries revealed that published probes (9, 30) hybridize to a variable region of the gene, so a new primer/probe set was designed to target a conserved region of this gene. This qPCR was coupled with an assay for *H. influenzae* that was developed to detect both typeable and nontypeable *H. influenzae* but not the closely related *Haemophilus haemolyticus* (38). Two microliters of DNA was used in each of two duplex qPCRs performed with Brilliant III Ultra-Fast QPCR master mix (Agilent Technologies) on a Stratagene Mx3005 real-time PCR instrument with an initial activation of 95°C for 3 min

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TABLE 1 Sequences and concentrations of primers and probes for duplex quantitative PCRs to detect *S. pneumoniae* and *S. aureus* (reaction 1) and *M. catarrhalis* and *H. influenzae* (reaction 2)

Reaction	Species and target gene	Primer and probe sequences[a] (5′–3′) [concn (nM)]	Reference and/or source
1	*S. pneumoniae lytA*	F, TCTTACGCAATCTAGCAGATGAAGC [96]; R, GTTGTTTGGTTGGTTATTCGTGC [96]; probe, FAM-TTTGCCGAAAACGCTTGATACAGGG-BHQ1 [96]	30
	S. aureus nuc	F, GTTGCTTAGTGTTAACTTTAGTTGTA [288]; R, GCACTATATACTGTTGGATCTTCAGAA [288]; probe, TxR-TGCATCACAAACAGATAACGGCGTAAATAGAAG-BHQ2 [192]	6, this study
2	*M. catarrhalis copB*	F, CGTGTTGACCGTTTTGACTTT [96]; R, TAGATTAGGTTACCGCTGACG [96]; probe, Cy5-AC CGACATCAACCCAAGCTTTGG-BHQ3 [96]	This study
	H. influenzae hpd	F, GGTTAAATATGCCGATGGTGTTG [96]; R, TGCATCTTTACGCACGGTGTA [288]; probe, HEX-TTGTGTACACTCCGT"T"GGTAAAAGAACTTGCAC-SpacerC3[b] [96]	38

[a] F, forward; R, reverse; FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1; TxR, Texas red; HEX, hexachloro-6-carboxyfluorescein.
[b] "T", BHQ-1 (dT).

followed by 40 cycles of 95°C for 20 s and 60°C for 20 s. Table 1 details PCR primers (Sigma-Aldrich) and dually labeled probes (Eurogentec).

qPCR data were used to determine the carriage rate (% positive) and carriage density (CFU/ml) for each bacterial species. Fisher's exact test and the Mann-Whitney test were used to evaluate differences in rate and density, respectively. P values <0.05 were considered statistically significant. Associations were measured by calculating odds ratios (OR) and 95% confidence intervals (CI 95%).

Of the 161 NP swabs examined, at least one pathogen was identified in 141 (87.5%). *M. catarrhalis* was identified most frequently, found in 122 (75.8%) swabs, followed by *S. pneumoniae* ($n = 92$; 57.1%) and *H. influenzae* ($n = 72$; 44.7%). The overall carriage rate of *S. aureus*, 3.3%, was consistent with reports from the Gambia (12) and Western Australia, Australia (39). *S. aureus* carriage is typically highest in neonates and older children (2, 22, 23), and the nose, rather than the nasopharynx, is the primary ecological niche (40). Due to its low frequency, *S. aureus* was not included in subsequent analyses. The higher colonization rates of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* were similar to those observed in high-risk populations (12, 16, 30, 39). Cocolonization was common, with 97 of 161 (60.2%) children colonized by multiple species compared with 44 (27.3%) colonized by one of the species tested. Positive associations between colonization by *S. pneumoniae* and *M. catarrhalis* (OR, 3.16; CI 95%, 1.49 to 6.71), *S. pneumoniae* and *H. influenzae* (OR, 3.85; CI 95%, 1.95 to 7.58), and *M. catarrhalis* and *H. influenzae* (OR, 6.70; CI 95%, 2.70 to 17.60) were found.

Regardless of 23vPPS booster status, vaccination with PCV7 did not affect carriage rates (Table 2) or densities (data not shown) of the four pathogens examined. The population of Fiji consists of 57% indigenous Fijians and 38% Indo-Fijians of Indian descent. The original vaccine trial was designed to approximate this breakdown in each vaccine group. As indigenous Fijian children are known to have higher rates of *S. pneumoniae* carriage (26), data were stratified by ethnicity and reanalyzed. Swabs from groups A, B, and H were collected from 99 Fijians and 54 Indo-Fijians (ethnicity self-reported upon enrollment). Indigenous Fijians (i) were more likely to carry two or more organisms (OR, 9.66; CI 95%, 4.49 to 20.77), (ii) had significantly higher carriage rates of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* (Table 2), and (iii) had higher carriage densities of *S. pneumoniae* and *M. catarrhalis* (Fig. 1) than Indo-Fijians.

Next, we examined the effects of pneumococcal vaccination separately for each ethnic group. No differences in carriage rates were observed (data not shown). Vaccination did not affect carriage densities of *S. pneumoniae* or *H. influenzae* in either indigenous Fijians or Indo-Fijians (Fig. 2A and B). Carriage densities of *M. catarrhalis* were significantly higher in indigenous Fijian children who received three doses of PCV7 plus a 23vPPV booster than in those who were unvaccinated or received PCV7 alone (Fig. 2C). This increase in *M. catarrhalis* density was not observed in Indo-Fijian children.

In summary, vaccination of Fijian infants with PCV7 alone or with the 23vPPS booster did not affect nasopharyngeal carriage rates or densities of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *S. aureus*. However, significant differences between the two major ethnic groups were found, with indigenous Fijians more likely to carry higher densities of multiple respiratory pathogens than Fijians of Indian descent.

Several studies investigated effects of pneumococcal vaccination on *S. aureus*, and most did not find any increases in *S. aureus* colonization associated with PCV7 (4, 5, 13) or PCV9 (14) vacci-

TABLE 2 Nasopharyngeal carriage rates of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in Fijian children stratified by vaccine group and ethnicity

Species	No. of positive samples/total no. of samples[a] (%) for:				
	Vaccine group				
	3 doses PCV7	3 doses PCV7 + 23vPPV	No vaccine	Fijian group	Indo-Fijian group
S. pneumoniae	30/54 (56)	29/48 (60)	33/59 (56)	68/99 (69)	20/54 (37)*
H. influenzae	24/54 (44)	24/48 (50)	24/59 (41)	66/99 (67)	6/54 (11)*
M. catarrhalis	42/54 (78)	39/48 (81)	41/59 (70)	91/99 (92)	24/54 (44)*

[a] *, $P < 0.001$. For all other comparisons, P was >0.05.

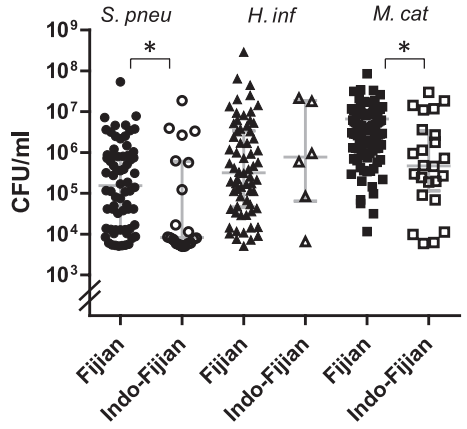

FIG 1 Nasopharyngeal carriage densities of *S. pneumoniae* (*S. pneu*), *H. influenzae* (*H. inf*), and *M. catarrhalis* (*M. cat*) in indigenous Fijian (Fijian) and Indo-Fijian infants. The median and interquartile ranges are shown in gray. *, $P < 0.05$. For all other comparisons, P was >0.05.

nation. In contrast, van Gils et al. reported a temporary increase in *S. aureus* colonization at 12 months of age in infants who received three doses of PCV7 (34). The low carriage rate of *S. aureus* in our study hindered our ability to assess the impact of pneumococcal vaccination on its carriage in Fijian infants. Reported increases in the proportion of otitis media caused by *H. influenzae* and *M. catarrhalis* associated with PCV7 vaccination (1, 4, 41) may reflect the reduction in cases caused by vaccine type *S. pneumoniae* rather than true increases in disease caused by other pathogens. For example, Stamboulidis et al. noted that, following PCV7 immunization, the overall rate of acute otitis media with otorrhea and the incidence of *S. pneumoniae*- and *H. influenzae*-caused otorrhea declined, yet *H. influenzae* replaced *S. pneumoniae* as the predominant organism (31).

Revai et al. examined nasopharyngeal colonization during acute otitis media and found an increase in *M. catarrhalis* in PCV7-vaccinated children compared with unvaccinated historical controls (24). Consistent with our findings, studies on the effects of pneumococcal vaccination in healthy children found no increases in carriage of *H. influenzae* (14, 35) or *M. catarrhalis* (35). We used the final swabs collected during the study, taken at 17 months, 14 months after the last dose of PCV7 and 5 months after the 23vPPS booster. Therefore, any transient effects of pneumococcal vaccination would not have been observed in this study.

We found strong positive associations between carriage of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*. Other researchers have reported similar associations between *S. pneumoniae* and *H. influenzae* (10, 12, 14), *S. pneumoniae* and *M. catarrhalis* (10, 12), and *M. catarrhalis* and *H. influenzae* (10, 37) in children of a comparable age. Pettigrew et al. (20) found negative associations between *S. pneumoniae* and *H. influenzae* and between *M. catarrhalis* and *H. influenzae* in children with upper respiratory infections, suggesting that the presence of an active infection may influence colonization dynamics. In our study, attributes of the host rather

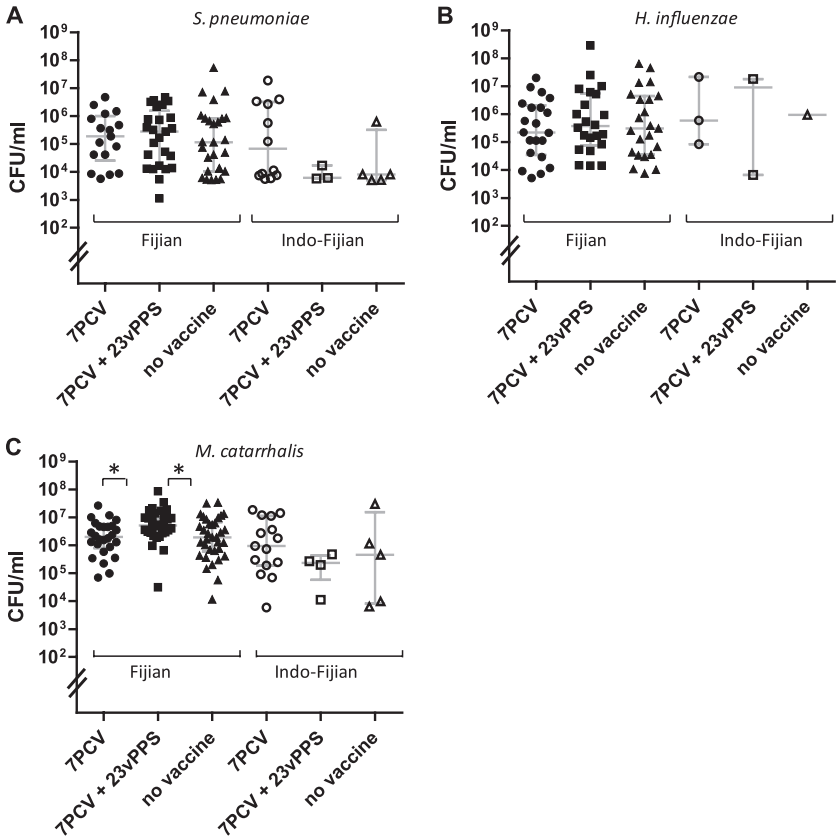

FIG 2 Nasopharyngeal carriage densities of *S. pneumoniae* (A), *H. influenzae* (B), and *M. catarrhalis* (C) separated by vaccine group and ethnicity. The median and interquartile ranges are shown in gray. *, $P < 0.01$. For all other comparisons, P was >0.05.

than the presence of a particular bacterial species seemed to be the dominant factor relating to the cocarriage of multiple pathogens. Host immunity likely plays a role in the negative association between *S. aureus* and *S. pneumoniae*, as this inverse relationship was not observed in HIV-positive children (14).

Indigenous Fijians have been shown to have higher incidence of pneumonia (15), invasive pneumococcal disease (28), and group A streptococcal disease (32) than Indo-Fijians. Reasons for this are unclear but may include genetic susceptibility, cultural practices, and/or environmental factors such as crowded living conditions. In our study, antibiotic use and monthly annual income levels were similar between the ethnicities but Fijian families had more children under 5 in the household, a risk factor for *S. pneumoniae* colonization (26). Differences in carriage between ethnic groups raise the possibility that vaccines targeting colonizing bacteria may have differential effects in different populations. Indeed, the 23vPPS booster was associated with increased carriage densities of *M. catarrhalis* in indigenous Fijians but not Indo-Fijians. Differences in susceptibilities to bacterial colonization warrant further investigation, and the high levels of carriage of multiple pathogens common in developing countries should be taken into consideration when designing and monitoring new pneumococcal vaccine introduction.

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